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Study of Abl1 tyrosine kinase inhibitors by liquid chromatography–electrospray ionization-mass spectrometry

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ABSTRACT

A method to study Abl1tyrosine kinase inhibitors (TKIs) by liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) was developed and validated. Chromatographic separation was achieved on a Symmetry® C-18 column using a gradient. The detection was performed by selected ion monitoring (SIM) mode via positive ESI interface. The limit of quantification (LOQ) was 40.8 nM for p-Abltide [product, KKGEAIpYAAPFA-NH_2] and 26.7 nM for Abltide (substrate, KKGEAIYAAPFA-NH_2). The residual plot of linearity calibration curve indicated a good fit with a linear model. Intra- and inter-day precision was less than 10% and accuracy was from −6.93% to +0.15%. Matrix effect was not significant in this method. The validated method was applied to an Abl1 TKIs study. Imatinib mesylate (IM) and dasatinib were used to evaluate this method and the IC_{50} values were 202.1 nM and 925.1 pM, respectively. Two natural products (−)- epigallocatechingallate (EGCG) and caffeic acid were tested with this model. The IC_{50} value of EGCG was found at 64.03 nM and caffeic acid showed fluctuant inhibitory activity from 26% to 55% in the concentration range from 1 nM to 1 mM. The IC_{50} value of a dimethylpyrrole hydroxylbenzoic acid derivative (MPB) was 1.915 μM.

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1. Introduction

Cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries. In 2008, about 12.7 million cancer cases and 7.6 million cancer deaths occurred worldwide [1].Cancer is a dynamic process that involves many complex factors [2], which may explain why a "magic bullet" cure has not been found [3]. The lack of such a cure leads to considerable attention being focused on chemoprevention as an alternative approach to the control of cancer [3]. Chronic Myeloid Leukemia (CML) is a type of cancer that starts in the blood-forming cells of the bone marrow and invades the blood. Based on the report of the American Cancer Society, CML accounts for about 20% of all leukemias and occurs at similar frequency in countries around the world. The age-adjusted incidence rate for CML was 1.6 per 100,000 adults [4]. Based on rates from 2000 to 2002, 1 in 619 men and women born today will be diagnosed with CML at some time during their lifetime, which shows the probability for a person to develop CML during his/her lifetime [5]. Both the incidence data and the lifetime risk estimates of CML shown here indicate that the attention paid on this disease is never too high.

CML was the first human malignant disease to be linked to a single, acquired genetic abnormality, which produces a constitutively active Bcr–Abl tyrosine kinase. The hybrid Bcr–Abl gene is formed by fusion between Abelson (Abl) tyrosine kinase gene at chromosome 9 and break-point cluster region (Bcr) gene at chromosome 22 [6]. This chromosomal translocation is called Philadelphia (Ph) chromosome. More than 90% of adults with CML are shown to be Ph chromosome positive (Ph+) [4]. CML is often divided into three phases, namely chronic phase, accelerated phase and blast crisis. Normally, drug treatment has more effect in the chronic phase. In the late 1980s, the Bcr–Abl gene was realized to be the most attractive target for CML therapy. Therefore, attempts to inhibit the tyrosine kinase activity of the constitutively active tyrosine kinase were made and a tyrosine kinase inhibitor, imatinib mesylate (IM), was discovered and developed for the treatment of CML during this process [7]. Now it is still the first-line therapy in patients with CML. However, some people with CML do not respond to IM and even individuals with responsive disease, who must remain on the drug indefinitely, can relapse [8,9]. Therefore, to surmount IM resistance, several other novel tyrosine kinase inhibitors (TKIs) were developed, such as dasatinib, nilotinib and bosutinib [10–12]. However, these agents are not efficacious in a small group of resistant patients [4]. It seems meaningful to further discover and develop more new, specific TKIs.

As the tyrosine kinase activity of the Bcr–Abl proteins is known to be essential to their transforming abilities [13–15],

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a specific inhibitor of Abl1 might be useful as a therapy for CML. Additionally, the Abl1 proto-oncogene has been implicated in processes of cell differentiation, cell division, cell adhesion and stress response. Deregulated Abl1is involved in cancer [16]. For these reasons, there is considerable interest in developing inhibitors that are capable of suppressing the activity of Abl1. Hence, in this study, Abl1 was used to investigate the activity of TKIs and potential TKIs in three groups. One group comprised two natural products and two commercial inhibitors were studied as the control group, whereas the last group contained some unknown compound.

Two of the potential inhibitors studied in this case were natural products. Historically, the majority of new drugs have been generated from natural products and from compounds derived from natural products. It seems that natural products represent privileged structures for drug discovery. This suggestion is supported by the fact that a limited number of protein folds are known now and that natural products must bind to some of them in order to be biosynthesized and to fulfill their inherent function in the producing organisms. Therefore, many of them may be structurally favored to bind to enzymes or protein receptors [17]. Polyphenols are a group of chemical substances found in plants that are available as chemoprotective agents against commonly occurring cancers. They are an important part of the human diet and are found in berries, grapes/wine, tea, chocolate/coca, coffee, soybeans and other fruits and vegetables [18]. (−)- epigallocatechingallate (EGCG) and caffeic acid are isolated from green tea and coffee, two important drinks for Eastern and Western populations, respectively. Currently, there is interest in the beneficial health effects of dietary polyphenols, because these compounds may have anti-oxidative, anti-inflammatory and anti-carcinogenic activities. Hence, these two classic polyphenols from diets were chosen as representatives to study their inhibitory activity on Abl1, in order to estimate whether CML could be controlled through dietary modification. EGCG is the most abundant catechin in tea and recent study suggests that it can inhibit xanthine oxidase activity to suppress intracellular ROS in HL-60 human promyelocytic leukemia cells [19]. Caffeic acid is a well-known phenolic acid present in many foods including coffee. A series of pull-down assays revealed that caffeic acid directly binds to Fyn kinase in an ATP non-competitive manner [20].

IM and dasatinib were used in this study as the control group. IM is an ATP-competitive inhibitor of the Abl protein kinase that is able to bind the inactive conformation of Bcr–Abl, preventing ATP from entering its binding pocket [21]. Dasatinib is a second generation tyrosine kinase inhibitor for IM resistant or intolerant Ph+ leukemias. It is a dual specific Src and Abl inhibitor that is able to bind and inhibit both active and inactive conformation of Abl, resulting in 100–300 fold higher activity than that of IM [11,22].

MPB, a derivative of compound 1 [4-(2,5 dimethyl-pyrrol-1-yl) -2-hydroxybenzoic acid], is able to inhibit the interaction of EphA4 with a peptide ligand as well as the natural ephrin ligands. It also inhibits ephrin-induced phosphorylation of EphA4 and EphA2 in cells, without affecting cell viability or the phosphorylation of other receptor tyrosine kinases [23]. Newly synthesized compound 1 does not show any detectable inhibitory activity. However, when left exposed to air at room temperature in a dry room, compound 1 acquires inhibitory activity of ephrin-A5-EphA4 binding. Through some yet unknown physical or chemical effects at room temperature, compound 1 acquires a darker brown color to become active MPB. Surprisingly, proton and carbon NMR spectra show no significant difference between these two compounds and the MS spectrum shows the same molecular weight [24]. The MS and NMR data of MPB can be found in the supplementary data of reference [24].

Robust methods that monitor enzyme activity and inhibitory potency are crucial to drug discovery and development. For high-throughput inhibitor screening, various forms of fluorescence and chemiluminescence readout always dominate the market. However, with the advance in sensitivity, speed and miniaturization of mass spectrometry methods, opportunities to couple mass spectrometry with screening will continue to come to the forefront [25]. Therefore, a LC–ESI-MS method for studying inhibitors of Abl1 is introduced in this report. Under the catalytic action of Abl1, a phosphate group can be transferred from an ATP to the substrate (Abltide) that results in a mass difference (80 Da) between Abltide and the product (p-Abltide). As such, the developed MS-based method can directly measure Abltide and p-Abltide in this case. For natural substrates without the chromophores required for spectrophotometric readouts, this ESI-MS approach dramatically reduces the reagent cost for derivatization and the false positive or false negative results caused by interference with the readout "tag". Thus, the establishment of an ESI-MS-based approach to measure inhibition of Abl1 is significant. However, many of the buffers and salts required for the enzyme reactions interfere with electrospray. Thus an online desalting system assisted by LC separation was applied in this study. By monitoring the conversion of substrate to product (instead of quantification vs. an internal standard), the need to use a costly heavy deuterated internal standard was eliminated. As described above, we developed a sensitive, accurate and cost effective LC–ESI-MS method and applied it to study the inhibitors of Abl1. The objective of this study was to establish a versatile and low cost LC–ESI-MS approach to discover new inhibitors of Abl1.

2. Experimental

2.1. Chemicals and reagents

Abl1 was purchased from proteinkinase.de (specific activity is 33.000 pmol/mg min; Biaffin GmbH & Co KG, Kassel, Germany). Abltide (KKGEAIYAAPFA-NH_2) was purchased from AnaSpec, Inc (San Jose, CA, USA). The phosphorylated Abltide [p-Abltide, KKGEAIpYAAPFA-NH_2] was synthesized by BACHEM (Bubendorf, Switzerland). IM was purchased from Cayman Chemical (Europe) and dasatinib was from LC Laboratories (Woburn, MA, USA). EGCG and caffeic acid were purchased from Sigma (St. Louis, MO, USA). MPB was provided by the Laboratory for Neurobiology, KU Leuven. Acetonitrile (LC/MS grade) and trifluoroacetic acid (TFA, ULC/MS grade) were obtained from Biosolve (Valkenswaard, the Netherlands). Dimethyl sulfoxide was from BASF (Antwerp, Belgium). Adenosine 5′-triphosphate disodium salt (ATP), DL-dithiothreitol (DTT) and anhydrous magnesium chloride were from Sigma-Aldrich (Bornem, Belgium). Tris[(hydroxymethyl) aminomethane] and Tris–HCl were purchased from AppliChem (Darmstadt, Germany). All solutions were prepared with Milli-Q water (Millipore, Bedford, MA, USA).

2.2. LC/MS system

High performance liquid chromatography (HPLC) was performed using a P680 LC pump from Dionex (Sunnyvale, CA, USA) and an autosampler AS100 Spectra Series with a fixed 20 μL loop from Thermo (San Jose, CA, USA). The LC system was coupled to a LCQ ion trap mass spectrometer (Thermo) with an ESI interface. Chromatographic separation was carried out using a Symmetry® C-18 column (150 × 2.1 mm i.d., particle size 5 μm;Waters, Massachusetts, USA) at room temperature (23 °C, MS room). Xcalibur 1.3 software (Thermo, California, USA) was used for instrument control, data acquisition and processing.

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